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Diversity, Divergence, and Evolution of Cell-Free Human Immunodeficiency Virus Type 1 in Vaginal Secretions and Blood of Chronically Infected Women: Associations with Immune Status

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Most human immunodeficiency virus type 1 (HIV-1) infections are believed to be the result of exposure to the virus in genital secretions. However, prevention and therapeutic strategies are usually based on characterizations of HIV-1 in blood. To understand better the dynamics between HIV-1 quasispecies in the genital tract and blood, we performed heteroduplex assays on amplified *env* products from cell-free viral RNA in paired vaginal secretion (VS) and blood plasma (BP) samples of 14 women followed for 1.5 to 3.5 years. Diversity and divergence were less in VS than in BP ($P = 0.03$ and $P < 0.01$, respectively), and divergence at both sites was correlated with blood $CD4^+$ cell levels (VS, $P = 0.05$; BP, $P = 0.01$). Evolution of quasispecies was observed in 58% of the women; the loss or gain of quasispecies in VS or BP was always accompanied by such changes at the other site. In addition, sustained compartmentalization of quasispecies in VS was found for four women, even as $CD4^+$ cell levels decreased to low levels (<50 cells/μl). Quasispecies changes over time were associated with fluctuations in $CD4^+$ cell levels; concordant increases or decreases in VS and BP divergence had greater $CD4^+$ cell level changes than intervals with discordant changes ($P = 0.05$), and women with evolving quasispecies had greater decreases in $CD4^+$ cell levels compared to that for women who maintained the same quasispecies ($P < 0.05$). Thus, diversity, divergence, and evolution of cell-free HIV-1 in VS can be different from that in BP, and dynamics between their respective quasispecies are associated with changes in $CD4^+$ cell levels.

The genome of human immunodeficiency virus type 1 (HIV-1) can undergo extensive diversification during the natural course of infection (6, 25, 37, 51). The genetic diversity of HIV-1 in an infected person, typically investigated in blood plasma, manifests itself as a collection of closely related but genetically distinct viral variants termed quasispecies. The genetic distances that distinguish these quasispecies are typically described as viral divergence. The generation of diversity and divergence among quasispecies is considered the genetic template that facilitates the evolution of HIV-1 that occurs during disease progression.

During the initial and chronic stages of infection, both the diversity and divergence of HIV-1 quasispecies in blood are believed to result from host-induced responses to viral infection, such as production of specific neutralizing antibodies from a patent immune system (1, 3, 38) and from viral responses to therapy with antiretroviral drugs (2, 11, 20, 29, 32). During the later stages of an infection, when the host immune response is in decline, the number of HIV-1 quasispecies circulating in the blood is reported to decrease, but these viral variants continue to evolve at the same or at an increased rate (53), possibly due, in part, to the preferential outgrowth of CXCR4-using viruses in some persons (23, 53). The majority of information about the dynamics of HIV-1 quasispecies has been collected through studies of proviral DNA or viral RNA in blood (2, 7, 14, 16, 17, 29, 34–36, 39, 42, 43, 53, 57), whereas much less has been reported on the diversity, divergence, and evolution of viruses shed in other tissues and organs, including the genital tracts of infected men and women (15, 31, 44, 46–48, 54, 56, 59).

The available information about HIV-1 replication in the genital tract of infected women suggests that most of the cell-free viral quasispecies shed in vaginal and cervical secretions are the product of local virus replication (18, 21). In addition, infected cells in the genital tract of HIV-1-positive women contain proviral quasispecies that can be phylogenetically and phenetically distinguished from those in peripheral blood mononuclear cells (21, 44, 48). These results indicate that infected cells in the genital tract of women contain compartmentalized proviral quasispecies that can produce the cell-free viral quasispecies in vaginal secretions that are reported to be different than those in the blood plasma (21, 31).

HIV-1 can be compartmentalized in the genital tract in women and men (18, 31, 46, 48, 59), but the influence of the host immune response on diversity, divergence, and evolution

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of viral quasispecies in these tissues is not well understood. Several studies found that cell-free virus levels in vaginal secretions are inversely correlated with $CD4^+$ cell levels in the blood of infected women (26, 30, 55), suggesting that a functioning immune system is associated with controlling viral replication in genital tissues. More recent evidence indicates that women with higher $CD4^+$ cell levels are more likely to have compartmentalized cell-free virus shed in their genital tract secretions (31). However, these studies did not address viral diversity and divergence in genital tract secretions compared to those in blood or changes (evolution) in genital tract quasispecies over time (31).

An in-depth analysis of proviral and cell-free viral quasispecies from blood suggests that changes in viral diversity and divergence can be associated with disease progression in HIV-1-infected men (53). However, the ability of HIV-1 to compartmentalize in the genital tract along with the likely influence of localized selection pressures in these tissues may produce viral diversities and divergences that are different from those in blood. HIV-1 proviral DNAs in cervical secretions and blood from recently infected women have different patterns of diversity and divergence between the two tissues (48). But proviral DNA from infected cells may harbor numerous defective or latent proviruses (49, 52) and may not reflect the population of cell-free quasispecies shed in genital secretions. Therefore, in this study we investigated the diversity, divergence, and evolution of cell-free HIV-1 shed in vaginal secretions compared to that in the blood of chronically infected women using heteroduplex assays that accurately assess those properties (16, 17). In addition, the association of the women's immune status over time with changes in vaginal and blood quasispecies was determined.

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MATERIALS AND METHODS

Study population. This analysis includes data from examinations of women enrolled in the Emory Vaginal Ecology Study of HIV infection. HIV-infected women were asked to enroll in this study if they were 18 to 49 years of age, had a normal Pap smear within the previous 12 months, were expected to live at least 1 year, and either were not on antiretroviral therapy or had taken the same antiretroviral therapy for at least 3 months prior to enrollment. Exclusion criteria were as described elsewhere (30). Participants were requested to refrain from vaginal intercourse and the use of intravaginal medications for 72 h before their examinations. Informed consent was obtained from the patients in this study, and the guidelines of the U.S. Department of Health and of the authors' institutions were followed in the conduct of clinical research.

Specimens. The samples analyzed in this study were matched vaginal secretion (VS) and blood plasma (BP) specimens that were collected at approximately 6-month or greater intervals from 14 women enrolled in the Emory Vaginal Ecology study. At each clinical exam, lavage samples were obtained by introducing 10 ml of phosphate-buffered saline into the vagina and collecting the pooled fluid in the posterior vaginal fornix. Vaginal secretions were tested for the presence of seminal fluid, as described elsewhere (13, 30). Endocervical swabs were obtained to culture *Neisseria gonorrhoeae* and to test for *Chlamydia trachomatis*. Wet mounts were examined to detect *Trichomonas vaginalis*. Women subsequently diagnosed with genital tract infections were treated for those infections. Venous blood was collected at each exam in a CPT Vacutainer tube that contained acid-citrate-dextrose anticoagulant. Methods for processing samples, assaying for blood contamination, and quantifying cell-free HIV-1 RNA were as described elsewhere (30, 41).

Cell-free HIV-1 RNA isolation, reverse transcription, and nested PCR. Cell-free HIV-1 was pelleted from 1-ml aliquots of cell-free VS and BP samples by centrifugation ($10^5 \times g$) for 30 min. RNA was extracted from the pellets, using the E.Z.N.A. Total RNA kit (Omega Bio-tek, Doraville, GA) and eluted in 50 μl of water according to the manufacturer's protocol. RNA extractions of VS and BP samples were performed at separate times to avoid the possibility of cross-contamination of matched samples during this procedure.

One-tenth of the extracted RNA (5 μl) was reverse-transcribed for 30 min at 42°C, using the GeneAmp RNA PCR kit (Perkin Elmer, Branchburg, NJ) and 50 pmol of an antisense primer (JH35R: 5′-CACTTCTCCAATTGTCCITCA-3′ [I indicates inosine]) (8). The resulting cDNA was used in a nested PCR (nPCR) protocol that amplifies a 540-bp fragment in the C2V4 region of the gp120 envelope gene (7002 to 7541 of HXB2) (8). The broadly reactive primer sets (JH35R and JH44F: 5′-ACAGTRCARTGYACACATGG-3′; JH33F: 5′-CTGT TIAATGGCAGICTAGC-3′; JH48R: 5′-ATGTATYCRCCTCCCATR-3′ [R indicates A or G, and Y indicates C or T]) and nested PCR cycling conditions were previously reported to amplify the C2V4 region from HIV-1 subtypes B, C, D, and E (8). Reverse transcription and PCR amplification of VS and BP samples were performed at separate times to avoid the possibility of cross-contamination of matched samples during this procedure.

To obtain a more accurate representation of the cell-free HIV-1 RNA quasispecies in a sample, three or more independent reverse transcription (RT)-nPCRs were performed for each VS and BP sample. Aliquots of the independent RT-nPCRs were pooled and used in subsequent analyses. In addition, the amount of amplifiable C2V4 template RNA in a sample was estimated, using a limiting-dilution protocol for the RT-nPCRs. These reactions were performed by use of a series of 10-fold dilutions (10^0 to 10^3) of the RNA extracts and the RT-nPCR described above. Amplifiable copies of C2V4 template RNA in the RT-nPCRs were estimated from the highest dilution of the RNA extract that produced a 540-bp amplicon detectable by ethidium bromide staining of a 1% agarose gel. Negative control RT-nPCRs were performed with water as an input template.

Diversity and divergence of HIV-1 in vaginal secretions and blood plasma. Diversity and divergence for cell-free HIV-1 quasispecies in VS and BP samples were determined using the heteroduplex mobility assay (HMA). For this study, diversity and divergence were estimated in the C2V4 region of the gp120 envelope by measuring differential electrophoretic mobilities of homo- and heteroduplexes of DNA formed within a population of RT-nPCR amplicons.

Five microliters of three individual RT-nPCRs from an RNA sample were combined and mixed with 1.5 μl of annealing buffer (1 M NaCl, 100 mM Tris-HCl, 20 mM EDTA). The PCR amplicons in the annealing buffer were denatured at 95°C for 3 min and placed immediately on ice for 20 min to promote heteroduplex formation. The samples were then electrophoresed on a 6% polyacrylamide gel at 100 V for 2 h and stained with ethidium bromide to visualize homo-and heteroduplex bands of DNA. Photographic images of the gels were made and used to record the migration distances of the fastest-moving homoduplex band and slower-moving heteroduplex bands. Gel photographs were scanned, saved as TIFF files, and imported into the Scion Image (v4.0.2) program (Scion Corporation, Frederick, MD). For each lane within a gel, signal intensities were recorded along the same distance (same number of pixels) starting just below the single-stranded DNA and ending just below the homoduplex band using the plot profile feature of the Scion Image program.

Normalized Shannon entropies, which reflect the amount of viral diversity in a sample (16), were calculated from pixel intensity pairs using the HDent program (available at the Los Alamos HIV sequence database web site, http://hiv-web.lanl.gov). In order to compare entropies between gels, each gel was standardized to the smallest number of pixels in the scans under study. Median mobility shifts (MMS), used to estimate the median divergence among quasispecies in a sample (16), were also determined for each lane using the HDent software. The maximum divergence among quasispecies in a sample was estimated by manually measuring the migration distance of the slowest-moving heteroduplex DNA band; this distance is reported as the maximum heteroduplex ratio, MHR (MHR = 1 − distance of slowest-moving heteroduplex band relative to distance of homoduplex band). Entropy, MMS, and MHR values range from 0 to 1; a value of 0 indicates no diversity/divergence (i.e., a homogeneous population), and values approaching 1 indicate greater diversity/divergence.

HIV-1 quasispecies evolution in vaginal secretions and blood plasma. The heteroduplex tracking assay (HTA) was used to evaluate the evolution of cell-free HIV-1 by following changes in quasispecies banding patterns of VS and BP over time. A unique HTA probe was generated for each woman by use of a cloned C2V4 RT-nPCR product derived from her BP HIV-1 RNA at the first clinic visit used for this study. Clonal plasmid DNA was extracted from a single bacterial colony containing a C2V4 RT-nPCR product in the pCR4-TOPO vector (Invitrogen, Carlsbad, CA). The plasmid DNA containing a C2V4 BP clone was used as a PCR template to generate a labeled HTA probe, using the

RT-nPCR primers JH33F and JH48R described above. In this PCR, the JH33F primer contained a 5′-6-carboxyfluorescein label that generated amplicons with one fluorescently labeled DNA strand.

A pool of driver DNA for an HTA consisted of six individual C2V4 RT-nPCRs that were combined before being precipitated, concentrated by lyophilization, and resuspended in 30 μl 10 mM Tris-Cl (pH 8.5). The concentrations of HTA driver and probe were estimated by comparing the intensities of ethidium bromide-stained bands after electrophoresis of 10-fold dilutions of each in 1.0% agarose gels. HTA hybridization reactions containing the fluorescently labeled C2V4 probe and 20-fold more unlabeled C2V4 driver DNA in annealing buffer (100 mM NaCl, 10 mM Tris-HCl, 2 mM EDTA, final concentrations) were heated to 95°C for 3 min and immediately placed on ice for 20 min. The HTA reactions were electrophoresed on a 6% polyacrylamide gel for 2 h at 100 V. The gels were scanned on the Storm 860 system (Molecular Dynamics, Sunnyvale, CA), using the excitation and emission wavelengths of 450 and 635 nm, respectively. The fluorescent gel images were viewed by use of the ImageQuant software program (Molecular Dynamics, Sunnyvale, CA). The 5′-6-carboxyfluorescein-labeled HTA probe without driver DNA was used as a negative control in the HTA.

Statistical analyses. Statistical analyses were performed by use of the Instat 3.0 software package (GraphPad Software, Inc, San Diego, CA). A P value of ≤ 0.05 was considered significant. Paired t tests and Spearman rank correlations were used to analyze HMA data of matched VS and BP samples. Observed distributions were examined by chi square tests. Spearman rank correlation coefficients were calculated to assess relationships between HMA data and clinical data, including blood $CD4^+$ cell counts and viral loads. Kruskal-Wallis tests and Dunn's multiple comparisons posttests were used to compare HMA data between multiple groups defined by $CD4^+$ cell levels. Mann-Whitney tests were performed to compare clinical data among patient groups defined by their HTA data. In addition, Fisher's exact test was used to compare distributions among different HTA-defined groups.

RESULTS

Study participants. For this analysis we examined 42 matched samples of VS and BP provided at 42 clinic visits of 14 HIV-1-infected women. For each woman, matched samples collected at three clinic visits were selected that spanned a total of 1.5 to 3.5 years. Fourteen of 28 (50%) intervals between clinic visits were 1 year (rounded to the nearest half year increment, all intervals ≥6 months) with four, six, three, and one interval at 0.5, 1.5, 2.0, and 2.5 years, respectively (Table 1). Each sample had a measurable HIV-1 *gag* RNA virus load that was determined by a quantitative RT-PCR assay, as described previously (30). The median virus load in VS samples was 5.7×10^4 total copies (range, 1.1×10^3 to 1.4×10^7 total copies) and the median BP virus load was 8.5×10^4 copies/ml (range, 4.6×10^3 to 7.4×10^5 copies/ml) (Table 1). The whole blood contribution to vaginal virus load did not exceed 1.6% in any of the lavage samples. Peripheral blood $CD4^+$ cell levels ranged from 1 to 532 cells/μl (median, 267 cells/μl). Four women were receiving antiretroviral therapy during all three clinic visits and another four women were receiving antiretroviral therapy at one or two clinic visits. *Trichomonas vaginalis* was detected at one clinic visit of two women, and *Chlamydia trachomatis* was detected at 1 clinic visit of another woman (Table 1). *Neisseria gonorrhea* and seminal fluid were not detected at any of the clinic visits.

HMA analysis of diversity and divergence of HIV-1 RNA quasispecies in vaginal secretions and blood. To avoid the possibility of underestimating HIV-1 quasispecies diversity and divergence due to low copy numbers of template RNA, samples with 10 or more amplifiable copies of *env* RNA (C2V4 region) were identified by limiting-dilution RT-nPCR. Twenty-eight (66%) of 42 VS samples and all of the BP samples had 10 or more amplifiable copies of input HIV-1 *env* RNA per RT-nPCR (Table 1, Fig. 1). These 28 matched samples were analyzed for viral RNA quasispecies diversity (entropy values), median divergence (MMS), and maximum divergence (MHR) using the C2V4 region in an HMA assay (Fig. 1).

Viral RNA diversity is usually lower in VS compared to matched BP. Viral RNA diversity was determined using the method for calculating Shannon entropy values for each HMA lane as described by Delwart et al. (16). The entropy values for diversity were compared between the VS and BP samples for each of the 28 clinic visits that had matched samples with adequate input viral RNA. Overall, viral diversity in VS was lower than in BP at 24 (86%) of the clinic visits (Fig. 2A-1). This greater number of clinic visits with lower levels of viral diversity in VS was statistically significant ($P < 0.01$). In addition, a comparison of the entropy values for all VS (mean, 0.84) and matched BP samples (mean, 0.90) showed that there was a significant difference between their viral RNA diversities ($P < 0.05$, Fig. 2B-1). Although the C2V4 RNA diversity was less in VS, the entropy values for matched samples of VS and BP were rank correlated ($r = 0.75$, $P < 0.0001$, Fig. 2C-1).

Viral RNA median and maximum divergence is usually less in VS compared to BP. Viral quasispecies divergence was analyzed using median mobility shifts (MMS) and maximum heteroduplex mobilities (MHR). The MMS value, as originally reported by Delwart et al. (16), is a calculated median of quasispecies divergence within a sample, whereas the MHR value reports the greatest level of divergence between quasispecies within a sample. MMS and MHR values for divergence were compared between the VS and BP samples for each of the 28 clinic visits that had matched samples with adequate input viral RNA. Median viral divergence in VS was lower than that in BP for 19 (68%) of the clinic visits (Fig. 2A-2). Maximum divergence was also lower in VS than BP for 17 (61%) of the clinic visits (Fig. 2A-3). The greater number of clinic visits with lower levels of median and maximum divergence in VS were statistically significant ($P < 0.01$ and $P < 0.05$, respectively). In addition, a comparison of the MMS values showed that median viral RNA divergence was significantly less in VS than in BP (mean MMS: VS = 0.26, BP = 0.32, $P < 0.001$, Fig. 2B-2). Although the median C2V4 RNA divergence was less in VS, the MMS values for matched samples of VS and BP were correlated ($r = 0.65$, $P < 0.001$, Fig. 2C-2). Similarly, analyses of the MHR values indicated that maximum divergence was less in VS than in BP (mean MHR: VS = 0.23, BP = 0.28, $P < 0.01$, Fig. 2B-3), and that VS and BP MHR values were correlated ($r = 0.60$, $P < 0.001$, Fig. 2C-3).

A systematic analysis of the impact of genital tract infections on quasispecies diversity and divergence was not possible since only three women (three clinic visits) had confirmed infections during the study (patients 022, 034, and 1007, Table 1). However, it was determined that viral quasispecies divergence in VS was equal or greater than that in BP for two and three of these clinic visits as measured by median and maximum divergence, respectively. In contrast, viral diversity in VS remained less than that in BP for these three matched samples.

Analyses of blood $CD4^+$ cell levels and HIV-1 RNA quasispecies diversity and divergence. To examine possible associations between a woman's immune status and viral diversity and divergence we calculated the correlations between blood

TABLE 1. Peripheral blood $CD4^+$ cell levels, virus loads, entropy, median mobility shift (MMS), and maximum heteroduplex ratio (MHR) values for blood plasma (BP) and vaginal secretions (VS), and occurrence of antiretroviral therapy and genital tract infections of 14 HIV-1-infected women at three clinic visits in ≤3.5 years (patients are grouped by mean $CD4^+$ cell levels)

Mean $CD4^+$	Patient no.	Time (yr)[a]	$CD4^+$ (cells/μl)	Virus load[b] $\log_{10}$ (BP, VS)	Entropy (BP, VS)	MMS (BP, VS)	MHR (BP, VS)	ART[c] or infection
<200 cells/μl	010		102	5.18, 4.78	0.21, 0.69	0.12, 0.14	0.16, 0.19	ART
		0.5	101	4.59, 3.64	0.96, 0.79	0.39, 0.16	0.32, 0.38	ART
		3	210	5.40, 5.05	0.91, 0.41	0.29, 0.07	0.08, 0	ART
	013		284	4.48, 5.21	0.93, 0.87	0.39, 0.18	0.31, 0.29	ART
		2	22	5.87, 5.30	0.93, 0.91*[d]	0.35, 0.33*	0.25, 0.25*	ART
		3	11	5.29, 4.70	0.89, 0.83	0.27, 0.30	0.19, 0.18	ART
	035		1	4.06, 3.10	0.91, 0.77	0.21, 0.13	0.20, 0	ART
		0.5	1	5.28, 5.33	0.93, 0.90	0.28, 0.20	0.32, 0	ART
		1.5	1	5.84, 3.67	0.96, 0.89	0.33, 0.20	0.32, 0.31	ART
	050		40	4.89, 4.21	0.90, 0.80*	0.25, 0.13*	0.26, 0*	
		1.5	10	4.44, 3.03	0.90, 0.84*	0.22, 0.21*	0.30, 0*	ART
		2	4	4.89, 4.37	0.88, 0.88	0.21, 0.18	0.33, 0.33	ART
	1005		237	4.80, 3.37	0.86, 0.67*	0.31, 0.13*	0.21, 0*	ART
		1.5	NA[e]	5.44, 5.32	0.86, 0.87	0.25, 0.26	0.19, 0.19	
		2.5	155	5.15, 4.58	0.91, 0.69*	0.30, 0.12*	0.19, 0*	
200–400 cells/μl	001		NA	4.86, 3.55	0.96, 0.89*	0.29, 0.20*	0.40, 0.08*	ART
		1.5	332	4.94, 4.27	0.97, 0.89*	0.34, 0.21*	0.35, 0.16*	ART
		2.5	270	4.76, 4.81	0.92, 0.89	0.22, 0.20	0.24, 0.23	
	014		277	4.84, 3.71	0.95, 0.89*	0.32, 0.22*	0.38, 0*	
		2	150	5.50, 4.95	0.95, 0.89	0.35, 0.27	0.32, 0.09	
		3.5	216	5.04, 3.84	0.96, 0.89*	0.40, 0.26*	0.23, 0.08*	
	053		491	4.65, 6.33	0.92, 0.79	0.33, 0.23	0.45, 0.20	
		1.5	378	5.61, 4.78	0.90, 0.87*	0.20, 0.18*	0.30, 0*	ART
		2.5	113	5.47, 4.88	0.95, 0.87	0.29, 0.20	0.31, 0.18	
	055		532	5.27, 4.73	0.96, 0.93	0.47, 0.47	0.38, 0.33	ART
		1	408	4.98, 4.20	0.96, 0.94	0.48, 0.37	0.40, 0.34	ART
		3	32	5.02, 4.98	0.96, 0.96	0.50, 0.53	0.41, 0.37	ART
	1007		263	3.86, 4.06	0.92, 0.85	0.29, 0.25	0.32, 0.18	
		1	240	3.66, 5.33	0.95, 0.91	0.31, 0.34	0.21, 0.24	*T. vaginalis*
		2	295	4.71, 4.28	0.93, 0.80	0.34, 0.26	0.21, 0.18	
	1012[d]		NA	5.57, 5.35	0.81, 0.70	0.21, 0.18	0.25, 0.09	
		1	275	5.25, 5.43	0.82, 0.75	0.23, 0.26	0.12, 0.12	ART
		1.5	NA	5.06, 4.88	0.90, 0.84	0.32, 0.34	0.23, 0.23	
>400 cells/μl	022		513	3.75, 3.42	0.81, 0.68*	0.17, 0.15*	0.25, 0.08*	
		1	483	4.33, 5.09	0.89, 0.82*	0.27, 0.20*	0.39, 0.15*	
		2	459	5.50, 5.33	0.95, 0.94	0.38, 0.40	0.25, 0.37	*C. trachomatis*
	034		433	4.45, 3.90	0.96, 0.91	0.44, 0.31	0.37, 0.31	
		1	449	4.57, 5.30	0.97, 0.96	0.49, 0.41	0.42, 0.42	*T. vaginalis*
		2	330	4.48, 4.26	0.97, 0.95*	0.48, 0.34*	0.42, 0.36*	
	045		467	4.66, 4.34	0.90, 0.76*	0.17, 0.10*	0.42, 0*	
		1	432	4.91, 7.15	0.94, 0.81	0.27, 0.14	0.34, 0.34	
		2.5	355	5.02, 5.61	0.91, 0.82	0.18, 0.17	0.31, 0.31	

[a] Time interval from first time point used in heteroduplex studies (rounded to nearest half-year increment; time between successive clinic visits ≥6 months).
[b] HIV-1 RNA copies/ml blood plasma or total HIV-1 RNA copies in cell-free vaginal lavage.
[c] ART, antiretroviral therapy.
[d] Patient grouped according to only available $CD4^+$ cell count.
[d] *, sample found to have <10 amplifiable copies of C2V4 RNA in limiting-dilution analysis.
[e] NA, not available.

$CD4^+$ cell levels and entropy, MMS, and MHR values. Blood $CD4^+$ cell levels were not correlated with viral diversity (entropy values) in either VS or BP. In contrast, $CD4^+$ cell levels were correlated with MMS and MHR values of VS samples ($r = 0.42$ and $P = 0.04$ and $r = 0.39$ and $P = 0.05$, respectively). For BP samples, only MHR values were correlated with $CD4^+$ cell levels ($r = 0.41$, $P = 0.01$). Viral diversity and divergence were further examined by grouping entropy, MMS, and MHR values into low (<200 cells/μl), medium (200 to 400 cells/μl), and high (>400 cells/μl) $CD4^+$ cell level categories. Although viral diversity was greatest in the high category for both VS and BP, the differences were not statistically significant (median entropies for low, medium, and high categories were: VS, 0.88, 0.84, and 0.93, respectively; and BP, 0.92, 0.93, and 0.94, respectively). Similarly, median divergence (MMS values) was also greatest in the high category for both VS and BP (median MMS values for low, medium, and high categories: VS, 0.20, 0.23, and 0.37, respectively; and BP, 0.28, 0.31, and 0.35, respectively), but these differences were also not significant. In contrast, maximum divergence (MHR values) in VS and BP were significantly different among the three $CD4^+$ cell level categories ($P < 0.05$, Fig. 3A; $P < 0.01$, Fig. 3B, respectively). For both VS and BP, maximum HIV-1 divergence was significantly greater in the high category (Fig. 3A and B).

There were no correlations between the virus loads

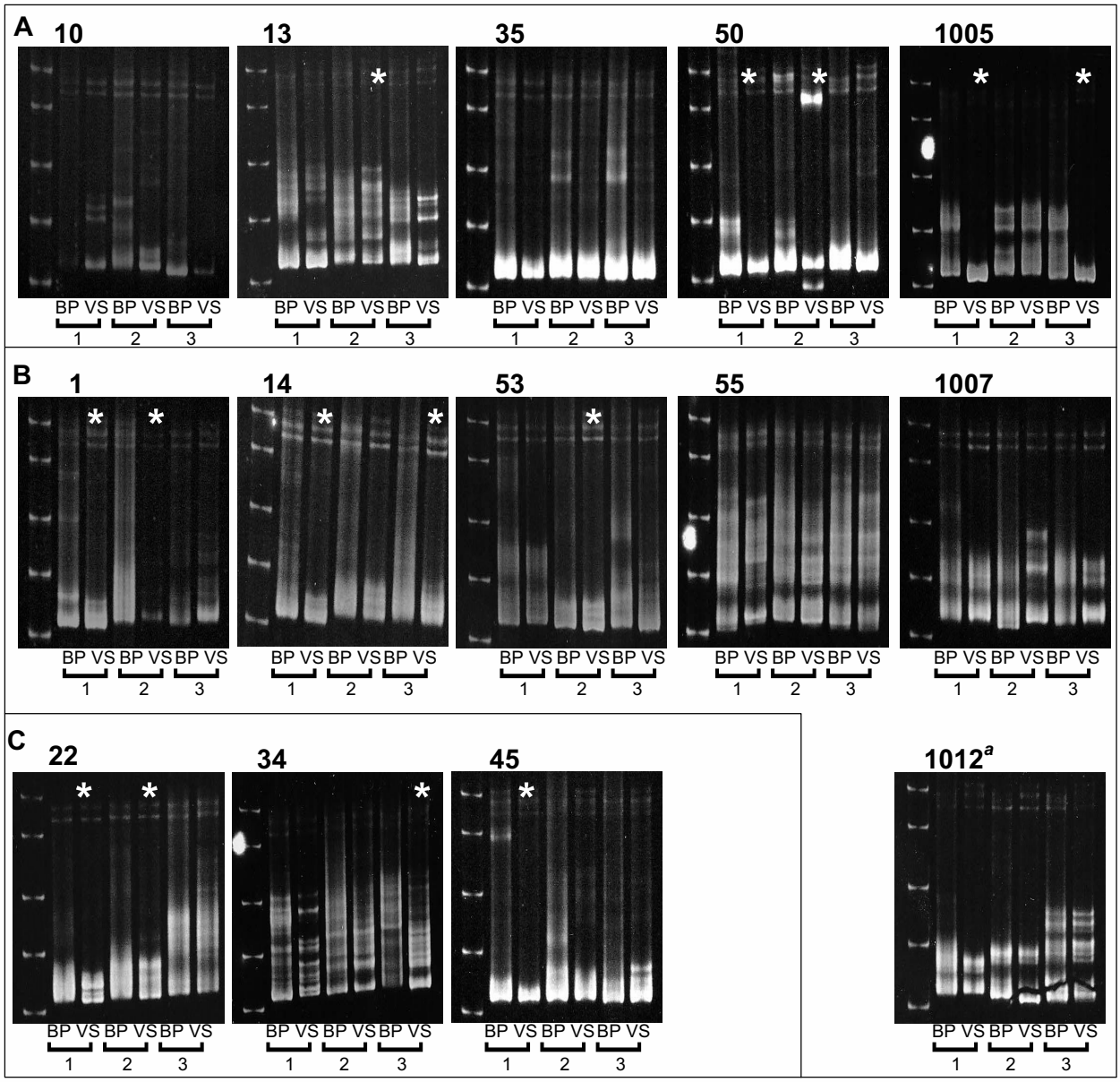

FIG. 1. HMA profiles of HIV-1 *env* (C2V4 region) quasispecies amplified from viral RNA in matched VS and BP collected at three clinic visits for each woman. HMA profiles are grouped according to a woman's mean peripheral blood $CD4^+$ cell level: (A) <200 cells/μl, (B) 200 to 400 cells/μl, and (C) >400 cells/μl. Lane 1 in each gel is a DNA ladder of 500, 700, 1,000, 1,500, and 2,000 base pairs. The bands between 1,500 and 2,000 base pairs in each HMA lane are single-stranded species that are not included in the HMA analysis. *, VS samples with <10 amplifiable copies of C2V4 RNA as determined by limiting-dilution analysis. [a], $CD4^+$-cell counts were available for only one clinic visit for this woman.

andHMA data (entropy, MMS, and MHR values) for VS or BP samples.

Changes in HIV-1 RNA quasispecies diversity and divergence over time, and associations with changes in $CD4^+$ cell levels. There were 14 intervals (clinic visits 1 to 2 and 2 to 3 or 1 to 3) in which all samples in an interval had ≥10 input copies of C2V4 RNA in their RT-nPCRs (Table 1). These 14 intervals were used to study changes in diversity (entropy values) and divergence (MMS and MHR values) in VS and BP quasispecies.

Time-dependent changes in HIV-1 quasispecies diversity and divergence. For the 14 available intervals, diversity and maximum divergence changes (entropy and MHR values) in VS samples were highly correlated with these changes in matched BP samples ($r = 0.80$ and $P = 0.0006$ and $r = 0.57$ and $P = 0.03$, respectively, Fig. 4A). This correlation was not found for changes in median divergence. For most intervals, matched VS and BP samples had a concordant increase or decrease in diversity, and median and maximum divergence (maximum divergence, see Fig. 4B). It was interesting that two of the five intervals that had discordant changes in maximum divergence were from one woman (patient 1007) who had a confirmed genital tract infection on her second sampling date (Table 1). In a comparison of the intervals, significantly greater changes

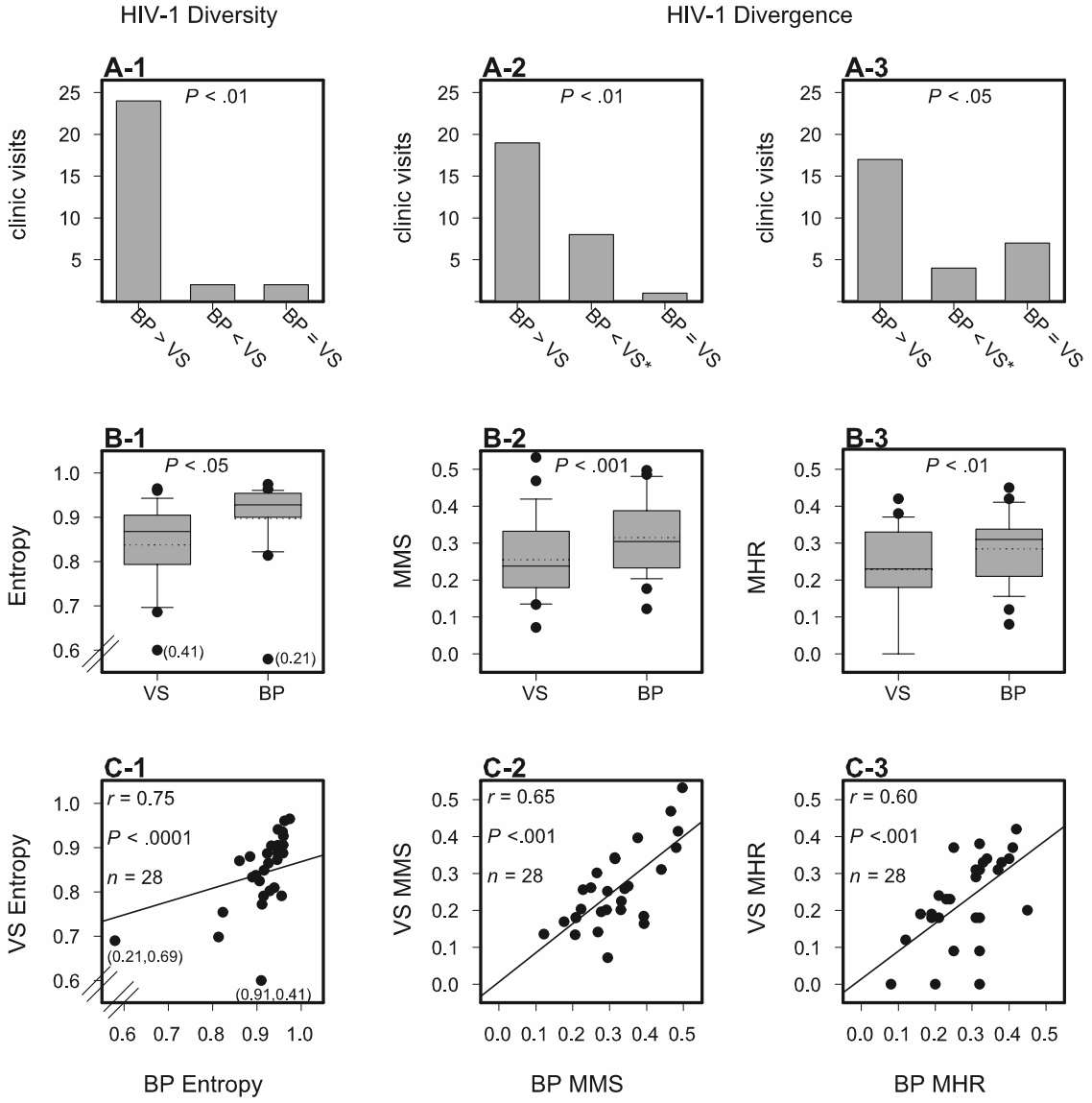

FIG. 2. Analyses of HIV-1 RNA (*env* C2V4) quasispecies diversity and divergence in matched VS and BP by HMA. (A) Number of clinic visits at which diversity (A-1), median divergence (A-2), and maximum divergence (A-3) were greater in BP (BP > VS) or in VS (BP < VS) or equal (BP = VS). *, Vaginal infections were diagnosed at two of these clinic exams. (B) Entropy (B-1), MMS (B-2), and MHR (B-3) values of VS and BP. Values within the 25% to 75% statistical quartiles are represented by grey boxes, and the solid and dashed lines through the boxes are the medians and means, respectively. Values within the 10% to 90% statistical intervals are represented by error bars, and outlier data are shown as solid circles. (C) Spearman rank correlations of entropy (C-1), MMS (C-2), and MHR (C-3) values between matched samples of VS and BP. In B-1 and C-1, entropy values in parentheses are for two outliers that lie beyond plot scales.

in $CD4^+$ cell levels occurred in those intervals that had a concordant increase or decrease in maximum divergence compared to those with discordant changes; median $CD4^+$ cell changes of 117 and 12, respectively ($P = 0.05$, Fig. 4B). These differences were not significant for diversity and median divergence changes.

A further analysis was done to determine if HIV-1 diversity and divergence were affected by menstrual cycle. We compared the HMA values in relation to the week of the menstrual cycle. None of the time points were collected during menses. Twenty-six time points were from women with regular menstrual cycles and another 16 time points were from women who previously had had hysterectomies. There were no significant differences of HMA values at different weeks in the menstrual cycle or from women who had had hysterectomies (all P values were ≥ 0.6).

HTA analysis of HIV-1 RNA quasispecies evolution in vaginal secretions and blood. (i) HTA sensitivity. Although HMA can analyze the pool of quasispecies for divergence within a sample, it is not designed to detect changes in specific viral variants over time. Therefore, we used HTA to detect changes in specific viral variants over time in a woman's VS and BP. The use of a labeled DNA probe, a C2V4 clone from the BP virus of each woman's first clinic visit, to form DNA heteroduplexes with the unlabeled target quasispecies in the HTA allowed us to detect the emergence or loss of specific viral variants from one clinic visit to the next. The performance of

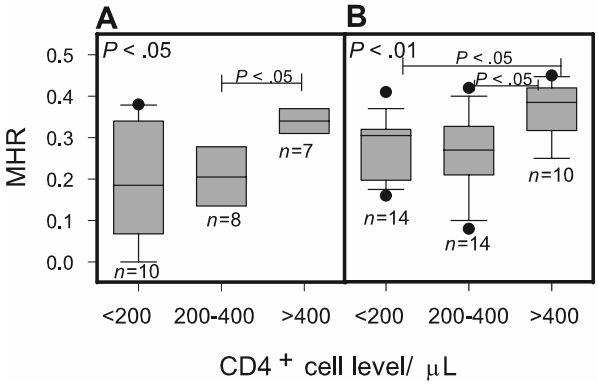

FIG. 3. Analyses of HIV-1 RNA (*env* C2V4) quasispecies maximum divergence in matched VS and BP with blood $CD4^+$ cell levels. Comparison of MHR values in VS (A) and BP (B) with low (<200 cells/μl), medium (200 to 400 cells/μl), and high (>400 cells/μl) $CD4^+$ cell levels. MHR values within the 25% to 75% statistical quartiles are represented by grey boxes, and the solid lines through the boxes are the medians. MHR values within the 10% to 90% statistical intervals are represented by error bars, and outlier data are shown as solid circles.

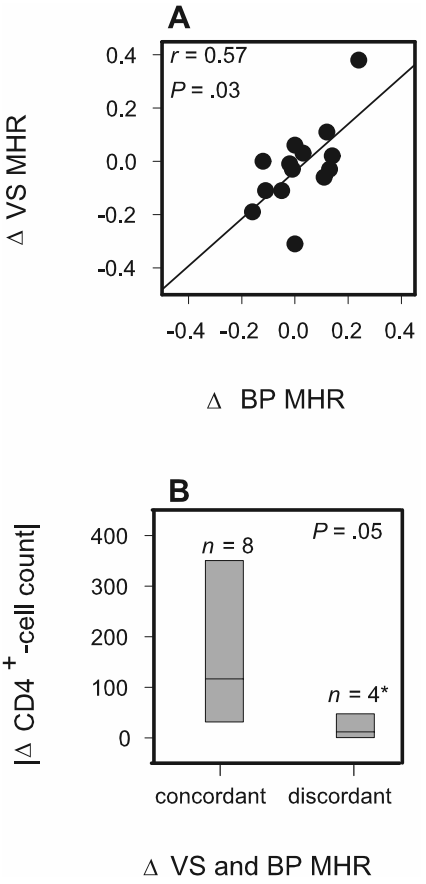

FIG. 4. Changes in the maximum divergence of HIV-1 RNA (*env* C2V4) quasispecies and $CD4^+$ cell levels. (A) Spearman rank correlation of changes in MHR values of matched VS and BP during a time interval. (B) Comparison of $CD4^+$ cell level changes during time intervals with concordant or discordant changes in MHR values between matched samples of VS and BP. $CD4^+$ cell level changes within the 25% to 75% statistical quartiles are represented by grey boxes, and the median changes are indicated by solid lines through boxes. $CD4^+$ cell level changes were unavailable for one interval in each group. *, A vaginal infection was diagnosed at a clinic visit included in two of these intervals during which discordant changes in divergence were observed.

the fluorescence-based HTA was determined by use of three C2V4 clones and the labeled probe from one woman. The probe alone, or the probe incubated with individual clones resulted in single, fluorescently labeled homoduplex and heteroduplex bands, respectively (Fig. 5A). When the HTA was performed using mixtures of the three clones in various proportions, a single clone comprising 5% of the mixture could be resolved (Fig. 5A). These results demonstrate that the HTA using fluorescently labeled, person-specific probes was capable of resolving the major C2V4 clonal sequences comprising 5% or more of the RT-nPCR-amplified quasispecies from cell-free HIV-1 RNA.

(ii) HIV-1 quasispecies evolution in vaginal secretions and blood plasma. Matched samples of VS and BP from 12 of the 14 women who had HMA data were available for HTA analysis (Fig. 5B to D). Eight (67%) of the 12 women had a total of 12 (50%) of 24 time intervals, in which all samples in an interval had ≥10 C2V4 RNA input copies in RT-nPCRs. Evolution of the major HIV-1 quasispecies, detected as the emergence or loss of HTA bands over time, occurred in a total of 9 (75%) of the 12 intervals of seven women (Fig. 5C and D). In all of these intervals, evolution of the major quasispecies in VS or BP samples was accompanied by evolving quasispecies in the other. However, two patterns of quasispecies evolution were detected in these women. In one type, HTA bands and changes in the bands over time in matched samples of VS and BP were identical or very similar at each interval (Fig. 5C). In contrast, four women had compartmentalized HTA banding patterns and evolutionary changes of the major quasispecies that were distinctly different between their matched VS and BP samples at each interval (Fig. 5D).

HIV-1 quasispecies evolution and blood $CD4^+$ cell levels. To examine the association of HIV-1 evolution with changes in immune status, $CD4^+$ cell levels and HTA patterns were analyzed. Declines in $CD4^+$ cell levels, determined from all available data, including data from clinic visits prior to or between the three collection times used in this study, were significantly greater for the seven women who had evolving quasispecies compared to the five women who maintained the same quasispecies populations for all time points (medians of −82 and −2 $CD4^+$ cells/μl/year, respectively; $P < 0.05$; Fig. 6). However, it should be noted that two women without evolving quasispecies populations had very low $CD4^+$ cell levels (<50 cells/μl) at all their clinic visits (patients 35 and 50, Fig. 6).

Although $CD4^+$ cell levels were higher for clinic visits at which compartmentalized quasispecies in VS was detected (Fig. 5D, Table 1), they were not significantly different from those clinic visits at which HTA patterns were similar (Fig. 5B and C, Table 1) (medians of 290 cells/μl and 150 cells/μl, respectively, $P > 0.05$). Antiretroviral therapy or changes in antiretroviral therapy, interval changes of HIV-1 RNA loads in VS or BP, and genital tract infections were not associated with HTA patterns or HIV-1 evolution.

DISCUSSION

The objectives of this analysis were to evaluate diversity, divergence, and evolution of cell-free viral RNA quasispecies

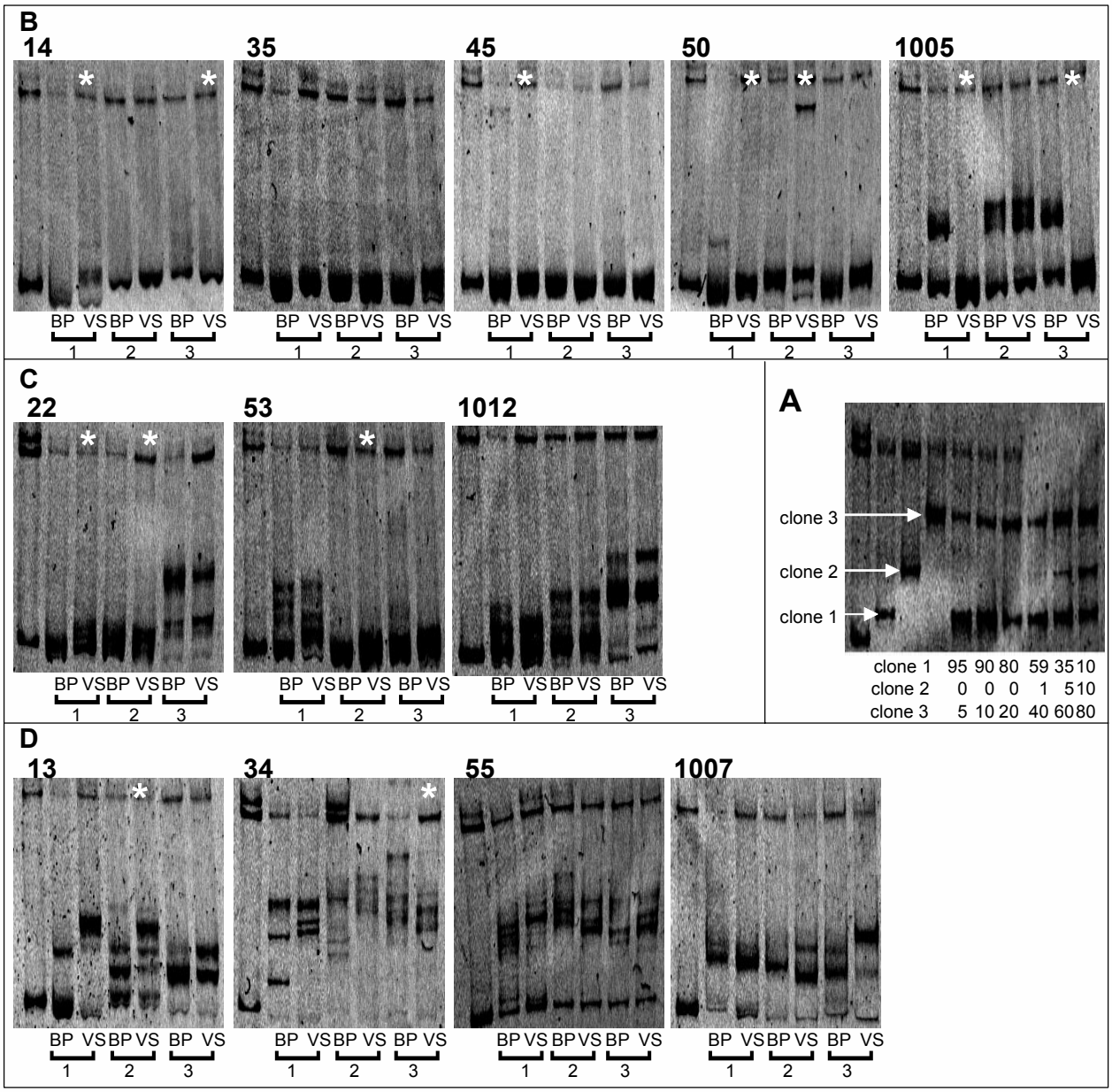

FIG. 5. HTA analysis of HIV-1 RNA (*env* C2V4) quasispecies evolution in VS and BP using a person-specific fluorescently labeled probe from BP. (A) Validation of the HTA using a cloned, person-specific probe from BP. The fluorescently labeled clone generated from *env* C2V4 RT-nPCR products of BP (from first clinic visit) was hybridized against itself (lane 1) or against other BP clones from clinic visits 1, 2, and 3 (clones from clinic visits 1, 2, and 3 are in lanes 2, 3, and 4, respectively). Clones from clinic visits 1, 2, and 3 were combined in various concentrations and hybridized with the fluorescent probe (lanes 5 to 10). Hybridized products were electrophoresed on a 6% polyacrylamide gel, and gel images were produced using a fluorescent scanner. Relative concentrations of each clone in a hybridization mixture are indicated for each lane. (B to D) HTA profiles of HIV-1 RNA (*env* C2V4) quasispecies of VS and BP collected at three clinic visits (1, 2, and 3) for each woman. A person-specific HTA probe was generated for each woman using a fluorescently labeled C2V4 PCR product from a single clone of the BP quasispecies at the first clinic visit. The first lane in all gel scans is the patient-specific probe alone, and the highest bands in all lanes are single-stranded species of the fluorescent probe. *, VS samples with <10 amplifiable copies of C2V4 RNA as determined by limiting-dilution analysis.

in the VS of women who had chronic HIV-1-infections. Furthermore, we examined the association of the quasispecies in VS samples with those in matched samples of BP and with peripheral blood $CD4^+$ cell levels, HIV-1 RNA levels, and genital tract infections. In this study, diversity and divergence were significantly less in VS than in BP HIV-1 populations. Our HMA findings showed that compared to matched blood virus, vaginal secretions had less viral diversity and divergence at most clinic visits and had overall lower entropy, MMS, and MHR values. A previous small cross-sectional study using HMA reported that HIV-1 proviral diversity was less in cervicovaginal secretions than in blood for four women (54).

Taken together, the results from our study and this previous report (54) indicate that in most instances, the pool of proviral

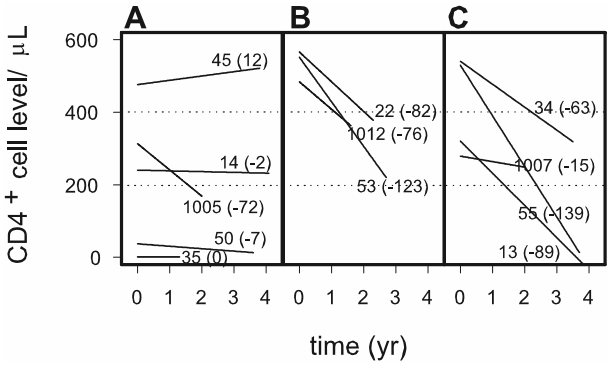

FIG. 6. Comparison of the linear regression profiles of $CD4^+$ cell levels over time of women with and without detectable evolution of the major HIV-1 RNA (*env* C2V4) quasispecies in VS and BP. (A) Women without detectable C2V4 evolution in matched VS and BP, (B) women with similar C2V4 evolution in VS and BP, and (C) women with different C2V4 evolution in VS and BP. Calculated $CD4^+$ cell declines/year are in parentheses following patient numbers.

quasispecies and the proviral population undergoing active replication in the female genital tract are genetically less diverse and divergent than those in blood; the likelihood of a more restricted pool of infected cells shedding cell-free HIV-1 into VS could account for these findings. Previous studies have shown that local replication is the principal source of HIV-1 in the female genital tract (18, 21). In contrast, cell-free HIV-1 quasispecies circulating in blood plasma are reported to originate from virus replication occurring at numerous sites of lymphoid tissues throughout the body (12, 22, 24, 28).

The correlations we observed between HIV-1 diversities in VS and BP and between HIV-1 divergences in quasispecies populations at these two sites suggest that one or more systemic factors were influencing HIV-1 quasispecies in both compartments. The correlations of $CD4^+$ cell levels in blood with viral divergence in both populations (VS, MMS and MHR; BP, MHR) indicate that immune status was an important systemic component that influenced HIV-1 genetics. In addition to these significant correlations, we observed the greatest diversity and divergence at higher $CD4^+$ cell levels (>400 cells/µl) in VS and BP samples, although statistical significance was not found. A previous study following HIV-1-infected men indicated a link between $CD4^+$ cell level and proviral quasispecies diversity and divergence in blood (53). These investigators reported that AIDS patients typically showed low proviral diversity and divergence, while patients who maintained high $CD4^+$ counts had higher diversity and divergence. Our data expand upon this observation in the provirus of men by showing that $CD4^+$ cell levels in chronically infected women impact the HIV-1 RNA quasispecies shed in their vaginal secretions.

Reports that show an association between a woman's $CD4^+$ cell level in blood and her virus loads in BP and VS also indicate that cellular immune activity affects the HIV-1 populations in these two compartments in a similar manner (26, 30, 55). In addition, a previous report of correlated HIV-1 *env*-specific immunoglobulin G titers in BP and cervicovaginal secretions of infected women (10) suggests that humoral anti-HIV-1 titers in VS and BP also could have contributed to the associations in viral diversities and divergences we observed. Future comparative studies that evaluate the antibody-dependent activities of virus neutralization and cellular cytotoxicity in VS and BP will be necessary to determine if both humoral and cellular immunity are significant factors in controlling quasispecies diversities and divergences in these two populations of HIV-1.

The correlation between changes of cell-free viral divergence in VS and BP over time provides further evidence that one or more systemic factors influence HIV-1 genetic distances in both compartments. In most women studied, greater changes in $CD4^+$ cell levels over time were associated with concordant increases or decreases of the maximum divergence in VS and BP. In contrast, we observed that women with relatively constant $CD4^+$ cell levels were more likely to have discordant changes in maximum divergence between VS and BP, suggesting that local factors, such as genital tract infections or local immunity (4, 5), could have been the overriding factors for influencing genital tract viral genetics when $CD4^+$ cell levels were stable.

Although a previous report found no association of increased proviral diversity with local inflammation (45), we found that the maximum genetic distances between quasispecies in cell-free virus in VS were abnormally high during vaginal infections, being greater or equal to those in matched BP. It is possible that mucosal infections, ulceration, and inflammation in the genital tract of HIV-1-infected women that are associated with increased shedding of cell-free virus in genital secretions (33, 58) also promote increases in cell-free viral divergence by activating virus replication from latently infected cells within the genital tract.

Our HTA data showed the emergence or loss of specific HIV-1 quasispecies in at least one interval for 58% of the women in our study. In these women, the observed evolution of VS or BP quasispecies was always accompanied by a simultaneous change in the other compartment during the same interval. Quasispecies evolution in only one compartment was never observed. Data from a previous study of HIV-1-infected women suggests that simultaneous evolutionary changes can occur in the proviral quasispecies of the genital tract and blood (48). However, the data include HIV-1 DNA genotypes that may not represent the actively replicating virus at these sites.

Even though HIV-1 evolution always occurred simultaneously in a woman's VS and BP, there were distinct differences in the major quasispecies between these two compartments at each time interval for four of seven women during which viral evolution was observed. These results indicate that compartmentalization of virus replication in the genital tract of these women persisted during the period of our study. Conversely, compartmentalization was not detected in the major quasispecies of VS in any of the time intervals of the other three women for whom viral evolutionary changes were observed. Although our assessment of compartmentalization did not use phylogenetic inferences, previous reports indicate that major differences between HTA banding patterns accurately represent quasispecies populations that are phylogenetically distinct (50, 60). Our findings are also supported by previous studies, which report that some, but not all, chronically infected women have cell-free HIV-1 with compartmentalized envelope sequences (21, 31). Our longitudinal analysis expands

upon recent cross-sectional findings (31) to further suggest that the absence or presence of compartmentalized HIV-1 envelope sequences in a woman's genital secretions is likely to be maintained over time, including periods when $CD4^+$ cell levels are low.

This study showed that the evolution of cell-free virus in VS and BP was associated with greater decreases in $CD4^+$ cell levels over time. For the women with no evolutionary changes, the HTA results indicated that the same limited number of major quasispecies persisted in their VS and BP over time. The lack of HIV-1 evolution in three of these women was likely associated with their consistently low $CD4^+$ cell levels throughout the study ($\approx$200 cells/μl and $<$50 cells/μl for one and two women, respectively). These results expand on the previous reports that showed changes in the quasispecies population of blood slows or becomes undetectable when $CD4^+$ cells have decreased to very low levels ($<$200 cells/μl) (53). In addition, this suggests that a woman with a chronically compromised immune system is more likely to maintain a homogeneous population of cell-free HIV-1 that is genetically similar between VS and BP. These data are the first information, to our knowledge, linking the evolution of cell-free quasispecies in genital secretions with the $CD4^+$ cell levels of chronically infected women.

Exposure to HIV-1 in genital secretions during sexual intercourse is thought to be the major mode of transmission worldwide (27, 40). Currently, there are differing reports as to whether multiple or single HIV-1 variants are transmitted sexually (14, 47, 59). Our data suggests that the highly variable levels of HIV-1 quasispecies diversity and divergence shed in a person's genital secretions may be one factor that determines whether single or multiple infectious viral variants are available and transmitted to a recipient. In addition, earlier sexual transmission studies, based on data from HIV-1 proviral quasispecies in the blood of documented virus donor and recipient pairs, suggests that transmission of HIV-1 through the genital mucosa was a highly restricted process, or bottleneck, that could select for certain minor viral variants circulating in blood (19).

Our data that some women shed distinctly different quasispecies in VS compared to BP and that these differences are maintained over time suggest that sexual transmission of distinct viral variants found in genital secretions may not involve selection mechanisms for minor quasispecies represented in BP. Furthermore, maternal genital secretions are believed to contain virus that can be perinatally transmitted to infants during a vaginal delivery (9). The report of an association between greater cell-free HIV-1 diversity in VS with perinatal transmission (45) shows that evaluations of viral quasispecies dynamics in genital secretions are necessary components for determining the viral and host-related mechanisms of transmission. The data from our study should help in interpreting how future immune and vaccine therapies could impact the natural course of HIV-1 shed in VS and how these changes are associated with a woman's immune status.

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REFERENCES

1. **Albert, J., B. Abrahamsson, K. Nagy, E. Aurelius, H. Gaines, G. Nystrom, and E. M. Fenyo.** 1990. Rapid development of isolate-specific neutralizing antibodies after primary HIV-1 infection and consequent emergence of virus variants which resist neutralization by autologous sera. AIDS **4:**107–112.
2. **Altfeld, M., E. S. Rosenberg, R. Shankarappa, J. S. Mukherjee, F. M. Hecht, R. L. Eldridge, M. M. Addo, S. H. Poon, M. N. Phillips, G. K. Robbins, P. E. Sax, S. Boswell, J. O. Kahn, C. Brander, P. J. R. Goulder, J. A. Levy, J. I. Mullins, and B. D. Walker.** 2001. Cellular immune responses and viral diversity in individuals treated during acute and early HIV-1 infection. J. Exp. Med. **193:**169–180.
3. **Arendrup, M., C. Nielsen, J.-E. S. Hansen, C. Pedersen, L. Mathiesen, and J. O. Nielsen.** 1992. Autologous HIV-1 neutralizing antibodies: emergence of neutralization-resistant escape virus and subsequent development of escape virus neutralizing antibodies. J. Acquir. Immune Defic. Syndr. **5:**303–307.
4. **Artenstein, A. W., T. C. VanCott, K. V. Sitz, M. L. Robb, K. F. Wagner, S. C. D. Veit, A. F. Rogers, R. P. Garner, J. W. Byron, P. R. Burnett, and D. L. Birx.** 1997. Mucosal immune responses in four distinct compartments of women infected with human immunodeficiency virus type 1: a comparison by site and correlation with clinical information. J. Infect. Dis. **175:**265–271.
5. **Belec, L., T. Dupre, T. Prazuck, C. Tevi-Benissan, J.-M. Kanga, O. Pathey, X.-S. Lu, and J. Pillot.** 1995. Cervicovaginal overproduction of specific IgG to human immunodeficiency virus (HIV) contrasts with normal or impaired IgA local response in HIV infection. J. Infect. Dis. **172:**691–697.
6. **Bonhoeffer, S., E. C. Holmes, and M. A. Nowak.** Causes of HIV diversity. Nature **376:**125, 1995.
7. **Brown, A. J. L.** 1997. Analysis of HIV-1 env gene sequences reveals evidence for a low effective number in the viral population Proc. Natl. Acad. Sci. USA **94:**1862–1865.
8. **Chen, J., N. L. Young, S. Subbarao, P. Warachit, S. Saguanwongse, S. Wongsheree, C. Jayavasu, C.-C. Luo, and T. D. Mastro.** 1999. HIV type 1 subtypes in Guangxi Province, China, 1996. AIDS Res. Hum. Retroviruses **15:**81–84.
9. **Chuachoowong, R., N. Shaffer, W. Siriwasin, P. Chaisilwattana, N. L. Young, P. A. Mock, S. Chearskul, N. Waranawat, T. Chaowanachan, J. Karon, R. J. Simonds, and T. D. Mastro.** 2000. Short-course antenatal zidovudine reduces both cervicovaginal human immunodeficiency virus type 1 RNA levels and risk of perinatal transmission. J. Infect. Dis. **181:**99–106.
10. **Chuachoowong, R., N. Shaffer, T. C. VanCott, P. Chaisilwattana, W. Siriwasin, N. Waranawat, N. Vanprapar, N. L. Young, T. D. Mastro, J. S. Lambert, and M. L. Robb.** 2000. Lack of association between human immunodeficiency virus type 1 antibody in cervicovaginal lavage fluid and plasma and perinatal transmission, in Thailand. J. Infect. Dis. **181:**1957–1963.
11. **Coffin, J. M.** 1995. HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy Science. **267:**483–489.
12. **Cohen, O. J., G. Pantaleo, M. Holodniy, S. Schnittman, M. Niu, C. Graziosi, G. N. Pavlakis, J. Lalezari, J. A. Bartlett, R. T. Steigbigel, J. Cohn, R. Novak, D. McMahon, and A. S. Fauci.** 1995. Decreased human immunodeficiency virus type 1 plasma viremia during antiretroviral therapy reflects downregulation of viral replication in lymphoid tissue. Proc. Natl. Acad. Sci. USA **92:**6017–6021.
13. **Davies, A.** 1978. A preliminary investigation using p-nitrophenyl phosphate to quantitate acid phosphatase on swabs examined in cases of sexual assault. Med. Sci. Law **18:**174–178.
14. **Delwart, E., M. Magierowska, M. Royz, B. Foley, L. Peddada, R. Smith, C. Heldebrant, A. Conrad, and M. Busch.** 2002. Homogeneous quasispecies in 16 out of 17 individuals during very early HIV-1 primary infection. AIDS **16:**189–195.
15. **Delwart, E. L., J. I. Mullins, P. Gupta, G. H. Learn, M. Holodniy, D. Katzenstein, B. D. Walker, and M. K. Singh.** 1998. Hum. immunodeficiency virus type 1 populations in blood and semen. J. Virol. **72:**617–623.
16. **Delwart, E. L., H. Pan, H. W. Sheppard, D. Wolpert, A. U. Neumann, B. Korber, and J. I. Mullins.** 1997. Slower evolution of human immunodeficiency virus type 1 quasispecies during progression to AIDS. J. Virol. **71:**7498–7508.
17. **Delwart, E. L., H. W. Sheppard, B. D. Walker, J. Goudsmit, and J. I. Mullins.** 1994. Human immunodeficiency virus type 1 evolution in vivo tracked by DNA heteroduplex mobility assays. J. Virol. **68:**6672–6683.
18. **De Pasquale, P. M., A. J. L. Brown, S. C. Uvin, J. Allega-Ingersoll, A. M. Caliendo, L. Sutton, S. Donahue, and R. T. D'Aquila.** 2003. Differences in HIV-1 pol sequences from female genital tract and blood during antiretroviral therapy. J. Acquir. Immune Defic. Syndr. **34:**37–44.
19. **Derdeyn, C. A., J. M. Decker, F. Bibollet-Ruche, J. L. Mokili, M. Muldoon, S. A. Denham, M. L. Heil, F. Kasolo, R. Musonda, B. H. Hahn, G. M. Shaw, B. T. Korber, S. Allen, and E. Hunter.** 2004. Envelope-constrained neutral-

ization-sensitive HIV-1 after heterosexual transmission. Science **303:**2018–2022.

20. **Doukhan, L., and E. Delwart.** 2001. Population genetic analysis of the protease locus of human immunodeficiency virus type 1 quasispecies undergoing drug selection, using a denaturing gradient-heteroduplex tracking assay. J. Virol. **75:**6729–6736.
21. **Ellerbrock, T. V., J. L. Lennox, K. A. Clancy, R. F. Schinazi, T. C. Wright, M. Pratt-Palmore, T. Evans-Strickfaden, C. Schnell, R. Pai, L. J. Conley, E. E. Parrish-Kohler, T. J. Bush, K. Tatti, and C. E. Hart.** 2001. Cellular replication of human immunodeficiency virus type 1 occurs in vaginal secretions. J. Infect. Dis. **184:**28–36.
22. **Erice, A., W. Li, H. H. Balfour, L. R. Boies, H. Melroe, and K. Henry.** 2001. Analysis of HIV-1 reverse transcriptase and protease sequences in paired plasma and lymphoid tissue specimens from HIV-1 infected individuals. AIDS **15:**831–836.
23. **Fenyo, E. M.** 2001. The role of virus biological phenotype in human immunodeficiency virus pathogenesis. AIDS Rev. **3:**157–168.
24. **Folkvord, J. M., D. M. Anderson, J. Arya, S. MaWhinney, and E. Connick.** 2003. Microanatomic relationships between CD8+ cells and HIV-1 producing cells in human lymphoid tissue in vivo. J. Acquir. Immune Defic. Syndr. **B:**469–476.
25. **Gao, F., Y. Chen, D. N. Levy, J. A. Conway, T. B. Kepler, and H. Hui.** 2004. Unselected mutations in human immunodeficiency virus type 1 genome are mostly nonsynonymous and often deleterious. J. Virol. **78:**2426–2433.
26. **Goulston, C., W. McFarland, and D. Katzenstein.** 1998. Human immunodeficiency virus type 1 RNA shedding in the female genital tract. J. Infect. Dis. **177:**1100–1103.
27. **Greenhead, P., P. Hayes, P. S. Watts, K. G. Laing, G. E. Griffin, and R. J. Shattock.** 2000. Parameters of human immunodeficiency virus infection of human cervical tissue and inhibition by vaginal virucides. J. Virol. **74:**5577–5586.
28. **Haase, A. T., K. Henry, M. Zupancic, G. Sedgewick, R. A. Faust, H. Melroe, W. Cavert, K. Gebhard, K. Staskus, Z.-Q. Zhang, P. J. Dailey, H. H. Balfour, A. Erice, and A. S. Perelson.** Quantitative image analysis of HIV-1 infection in lymphoid tissue. Science **274:**985–989, 1996.
29. **Hance, A. J., V. Lemiale, J. Izopet, D. Lecossier, V. Joly, P. Massip, F. Mammano, D. Descamps, F. Brun-Vezinet, and F. Clavel.** 2001. Changes in human immunodeficiency virus type 1 populations after treatment interruption in patients failing antiretroviral therapy. J. Virol. **75:**6410–6417.
30. **Hart, C. E., J. L. Lennox, M. Pratt-Palmore, T. C. Wright, R. F. Schinazi, T. Evans-Strickfaden, T. J. Bush, C. Schnell, L. J. Conley, K. A. Clancy, and T. V. Ellerbrock.** 1999. Correlation of human immunodeficiency virus type 1 RNA levels in blood and the female genital tract. J. Infect. Dis. **179:**871–882.
31. **Kemal, K. S., B. Foley, H. Burger, K. Anastos, H. Minkoff, C. Kitchen, S. M. Philpott, W. Gao, E. Robison, S. Holman, C. Dehner, S. Beck, W. A. Meyer, A. Landay, A. Kovacs, J. Bremer, and B. Weiser.** 2003. HIV-1 in genital tract and plasma of women: compartmentalization of viral sequences, coreceptor usage, and glycosylation. Proc. Natl. Acad. Sci. USA **100:**12972–12977.
32. **Kijak, G. H., V. Simon, P. Balfe, J. Vanderhoeven, S. E. Pampuro, C. Zala, C. Ochoa, P. Cahn, M. Markowitz, and H. Salomon.** 2002. Origin of human immunodeficiency virus type 1 quasispecies emerging after antiretroviral treatment interruption in patients with therapeutic failure. J. Virol. **76:**7000–7009.
33. **Lawn, S. D., S. Subbarao, T. C. Wright, T. Evans-Strickfaden, T. V. Ellerbrock, J. L. Lennox, S. T. Butera, and C. E. Hart.** 2000. Correlation between human immunodeficiency virus type 1 RNA levels in the female genital tract and immune activation associated with ulceration of the cervix. J. Infect. Dis. **181:**1950–1956.
34. **Learn, G. H., D. Muthui, S. J. Brodie, T. Zhu, K. Diem, J. I. Mullins, and L. Corey.** 2002. Virus population homogenization following acute human immunodeficiency virus type 1 infection. J. Virol. **76:**11953–11959.
35. **Liu, S.-L., T. Schacker, L. Musey, D. Shriner, M. J. McElrath, L. Corey, and J. I. Mullins.** 1997. Divergent patterns of progression to AIDS after infection from the same source: human immunodeficiency virus type 1 evolution and antiviral responses. J. Virol. **71:**4284–4295.
36. **Malaspina, A., S. Moir, D. C. Nickle, E. T. Donoghue, K. M. Ogwaro, L. A. Ehler, S. Liu, J. A. M. Mican, M. Dybul, T.-W. Chun, J. I. Mullins, and A. S. Fauci.** 2002. Human immunodeficiency virus type 1 bound to B cells: relationship to virus replicating in CD4+ T cells and circulating in plasma. J. Virol. **76:**8855–8863.
37. **Malim, M. H., and M. Emerman.** HIV-1 sequence variation: drift, shift, and attenuation. Cell. **104:**469–472, 2001.
38. **McKeating, J. A., J. Gow, J. Goudsmit, L. H. Pearl, C. Mulder, and R. A. Weiss.** Characterization of HIV-1 neutralization escape mutants. AIDS **3:**777–784, 1989.
39. **Menu, E., F.-X. M'bopi Keou, S. Lagaye, S. Pissard, P. Mauclere, G. Scarlatti, J. Martin, M. Goossens, G. Chaouat, and F. Barre-Sinoussi.** 1999. Selection of maternal human immunodeficiency virus type 1 variants in human placenta J. Infect. Dis. **179:**44–51.
40. **Mostad, S. B., and J. K. Kreiss.** Shedding of HIV in the genital tract. AIDS **10:**1305–1315, 1996.
41. **Mulder, J., N. McKinney, C. Christopherson, J. Sninsky, L. Greenfield, and S. Swok.** 1994. A rapid and simple PCR assay for quantitation of human immunodeficiency virus type 1 RNA in plasma: application to acute retroviral infection. J. Clin. Microbiol. **32:**292–300.
42. **Nelson, J. A. E., F. Baribaud, T. Edwards, and R. Swanstrom.** 2000. Patterns of changes in human immunodeficiency virus type 1 V3 sequence populations late in infection. J. Virol. **74:**8494–8501.
43. **Nowak, M. A., R. M. Anderson, M. C. Boerlijst, S. Bonhoeffer, R. M. May, and A. J. McMichael.** HIV-1 evolution and disease progression. Science **274:**1008–1011, 1996.
44. **Overbaugh, J., R. J. Anderson, J. O. Ndinya-Achola, and J. K. Kreiss.** 1996. Distinct but related human immunodeficiency virus type 1 variant populations in genital secretions and blood. AIDS Res. Hum. Retroviruses **12:**107–115.
45. **Panther, L. A., L. Tucker, C. Xu, R. E. Tuomala, J. I. Mullins, and D. J. Anderson.** 2000. Genital tract human immunodeficiency virus type 1 (HIV-1) shedding and inflammation and HIV-1 env diversity in perinatal HIV-1 transmission. J. Infect. Dis. **181:**555–563.
46. **Ping, L.-H., M. S. Cohen, I. Hoffman, P. Vernazza, F. Seillier-Moiseiwitsch, H. Chakraborty, P. Kazembe, D. Zimba, M. Maida, S. A. Fiscus, J. J. Eron, R. Swanstrom, and J. A. E. Nelson.** 2000. Effects of genital tract inflammation on human immunodeficiency virus type 1 V3 populations in blood and semen. J. Virol. **74:**8946–8952.
47. **Poss, M., H. L. Martin, J. K. Kreiss, L. Granville, B. Chohan, P. Nyange, K. Mandaliya, and J. Overbaugh.** 1995. Diversity in virus populations from genital secretions and peripheral blood from women recently infected with human immunodeficiency virus type 1. J. Virol. **69:**8118–8122.
48. **Poss, M., A. G. Rodrigo, J. J. Gosink, G. H. Learn, D. De Vange Panteleeff, H. L. Martin, J. Bwayo, J. K. Kreiss, and J. Overbaugh.** 1998. Evolution of envelope sequences from the genital tract and peripheral blood of women infected with clade A human immunodeficiency virus type 1. J. Virol. **72:**8240–8251.
49. **Psallidopoulos, M. C., S. M. Schnittman, L. M. Thompson, M. Baseler, A. S. Fauci, H. C. Lane, and N. P. Salzman.** 1989. Integrated proviral human immunodeficiency virus type 1 is present in $CD4^+$ peripheral blood lymphocytes in healthy seropositive individuals. J. Virol. **63:**4626–4631.
50. **Quinones-Mateu, M. E., J. L. Albright, A. Mas, V. Soriano, and E. J. Arts.** 1998. Analysis of pol gene heterogeneity, viral quasispecies, and drug resistance in individuals infected with group O strains of human immunodeficiency virus type 1. J. Virol. **72:**9002–9015.
51. **Saag, M. S., B. H. Hahn, J. Gibbons, Y. Li, E. S. Parks, W. P. Parks, and G. M. Shaw.** Extensive variation of human immunodeficiency virus type 1 in vivo. Nature **334:**440–444, 1988.
52. **Sanchez, G., X. Xu, J.-C. Chermann, and I. Hirsch.** 1997. Accumulation of defective viral genomes in peripheral blood mononuclear cells of human immunodeficiency virus type 1-infected individuals. J. Virol. **71:**2233–2240.
53. **Shankarappa, R., J. B. Margolick, S. J. Gange, A. G. Rodrigo, D. Upchurch, H. Farzadegan, P. Gupta, C. R. Rinaldo, G. H. Learn, X. He, X.-L. Huang, and J. I. Mullins.** 1999. Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection. J. Virol. **73:**10489–10502.
54. **Sutthent, R., K. Sumrangsurp, P. Wirachsilp, P. Chaisilwattana, A. Roongpisuthipong, P. Chaiyakul, P. Nooma, M. Honda, and P. Warachit.** 2001. Diversity of HIV-1 subtype E in semen and cervicovaginal secretion. J. Hum. Virol. **4:**260–268.
55. **Uvin, S. C., and A. M. Caliendo.** 1997. Cervicovaginal human immunodeficiency virus secretion and plasma viral load in human immunodeficiency virus-seropositive women. Obstet. Gynecol. **90:**739–743.
56. **Verhofstede, C., E. Demecheleer, N. De Cabooter, P. Gaillard, F. Mwanyumba, P. Claeys, V. Chohan, K. Mandaliya, M. Temmerman, and J. Plum.** 2003. Diversity of the human immunodeficiency virus type 1 (HIV-1) env sequence after vertical transmission in mother-child pairs infected with HIV-1 subtype A. J. Virol. **77:**3050–3057.
57. **Wolinsky, S. M., B. T. M. Korber, A. U. Neumann, M. Daniels, K. J. Kunstman, A. J. Whetsell, M. R. Furtado, Y. Cao, D. D. Ho, J. T. Safrit, and R. A. Koup.** 1996. Adaptive evolution of human immunodeficiency virus-type 1 during the natural course of infection. Science **272:**537–542.
58. **Wright, T. C., S. Subbarao, T. V. Ellerbrock, J. L. Lennox, T. Evans-Strickfaden, D. G. Smith, and C. E. Hart.** 2001. Human immunodeficiency virus type 1 expression in the female genital tract in association with cervical inflammation and ulceration. Am. J. Obstet. Gynecol. **184:**279–285.
59. **Zhu, T., N. Wang, A. Carr, D. S. Nam, R. Moor-Jankowski, D. A. Cooper, and D. D. Ho.** 1996. Genetic characterization of human immunodeficiency virus type 1 in blood and genital secretions: evidence for viral compartmentalization and selection during sexual transmission. J. Virol. **70:**3098–3107.
60. **Zhu, T., N. Wang, A. Carr, S. Wolinsky, and D. D. Ho.** 1995. Evidence for coinfection by multiple strains of human immunodeficiency virus type 1 subtype B in an acute seroconverter. J. Virol. **69:**1324–1327.